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PRO-C3, a Serological Marker of Fibrosis, During Childhood and Correlations With Fibrosis in Pediatric NAFLD

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Nonalcoholic fatty liver disease (NAFLD) is a common chronic liver disease in children and may lead to cirrhosis requiring liver transplant. Thus, prompt diagnosis of advanced fibrosis is essential. Our objectives were to examine PRO-C3 (a neo-epitope pro-peptide of type III collagen formation) levels across childhood/adolescence and associations with advanced fibrosis in pediatric NAFLD. This cross-sectional study included 88 children and adolescents with biopsy-proven NAFLD (mean age: 13.9 ± 2.9 years, 71% male) and 65 healthy participants (11.8 ± 4.5 years, 38% male). PRO-C3, and the bone remodeling biomarkers C-terminal telopeptide of type I collagen (CTX-I; bone resorption) and osteocalcin (N-MID; bone formation), were measured in serum by enzyme-linked immunosorbent assay. Fibrosis was assessed by liver biopsy in participants with NAFLD, who were categorized as having advanced (Ishak score ≥ 3) or none/mild fibrosis (Ishak score ≤ 2). Overall, PRO-C3 was similar in participants with NAFLD (median [interquartile range]: 20.6 [15.8, 25.9] ng/mL) versus healthy participants (19.0 [13.8, 26.0] ng/mL), but was significantly lower in older adolescents ≥ 15 years old (16.4 [13.0, 21.2] ng/mL) compared with children ≤ 10 years old (22.9 [18.1, 28.4] ng/mL; $P < 0.001$) or 11-14 years old (22.4 [18.3, 31.2] ng/mL; $P < 0.001$). PRO-C3 was also directly correlated with levels of CTX-I and N-MID ($r = 0.64$ and $r = 0.62$, respectively; both $P < 0.001$). Among participants with NAFLD, PRO-C3 was higher in those with advanced fibrosis (median [IQR]: 28.5 [21.6, 37.6]) compared with none/mild fibrosis (20.3 [18.2, 22.8]; $P = 0.020$) in models adjusted for age, sex, and body mass index *z*-score. However, associations were attenuated after additionally adjusting for bone-remodeling CTX-I ($P = 0.09$) or N-MID ($P = 0.08$). *Conclusion:* Collectively, these findings show that PRO-C3 levels are higher in children with advanced fibrosis in NAFLD, but are also influenced by age and pubertal growth spurt, assessed by bone remodeling biomarkers, and therefore may not be a reliable biomarker for liver fibrosis in pediatric NAFLD until late adolescence. (*Hepatology Communications* 2021;5:1860-1872).

In the United States, one of three children are either obese or overweight.[1] From this population, almost 34% have nonalcoholic fatty liver disease (NAFLD),[2] which is currently the most common cause of liver disease in children. NAFLD is characterized by the accumulation of fat in the liver

Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; CI, confidence interval; CTX-I, C-terminal telopeptide of type I collagen; ECM, extracellular matrix; LS-mean, least-squares mean; N-MID, osteocalcin; NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; NASH CRN, NASH Clinical Research Network.

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(hepatic steatosis) and can progress to a more severe form of the disease called nonalcoholic steatohepatitis (NASH), characterized by portal and/or lobular inflammation with hepatocyte injury. A few natural history studies have shown that children with NASH have low-grade chronic tissue inflammation and over time can develop progressive fibrosis and other comorbidities, especially type 2 diabetes, and may eventually require liver transplantation.[3,4]

This potential progression of NAFLD to fibrosis and cirrhosis in children and young adults is concerning, given evidence that advanced fibrosis may be the most crucial histologic feature in predicting mortality in patients with NAFLD.[5,6] Liver biopsy historically stands as the gold standard for diagnosing liver fibrosis; however, this is an invasive and expensive procedure prone to sampling error.[7] Therefore, several fibrosis scoring systems based on routine laboratory and/or clinical measures have been proposed, but none have proven to be as valid in children as in adults.[8]

Fibrogenesis is based on remodeling characterized by the excess production and deposition of extracellular matrix (ECM). This dynamic process leads to the constant release of proteins related to degradation or formation of ECM.[9] PRO-C3, a neo-epitope pro-peptide of type III collagen formation, has been shown to be a strong and independent predictor of fibrosis stage in adults with NAFLD.[10,11] In longitudinal studies in adults, change in PRO-C3 over time has been shown to correlate with histological changes in fibrosis.[12,13] Furthermore, in recent clinical trials, PRO-C3 has shown promising results as a biomarker to monitor fibrosis response to therapy.[14-17]

Because of the high prevalence of pediatric NAFLD and risks associated with progression to advanced fibrosis, there is a critical need to develop noninvasive methods for screening, diagnosing, and monitoring fibrosis in children and adolescents with NAFLD. In this study, our objectives were to describe PRO-C3 levels in a cross-sectional sample of children and adolescents with and without NAFLD in relation to age, sex, race/ethnicity, weight status, and the pubertal growth spurt (measured through the surrogate markers of bone turnover C-terminal telopeptide of type I collagen [CTX-I] and osteocalcin [N-MID]),[18] and examine whether PRO-C3 differs in children with NAFLD and advanced fibrosis compared with NAFLD and none/mild fibrosis or healthy children.

Patients and Methods

STUDY POPULATION

The initial study consisted of a cohort of 260 children who underwent liver biopsy at Children's Healthcare of Atlanta from 2006 to 2020 as part of the Emory Liver Biopsy Data Biorepository.[19,20] Among these, 93 children were eligible for the present study based on have a stored blood sample for analysis and a NAFLD diagnosis by liver biopsy (steatosis > 5%); other etiologies were excluded by standard clinical, laboratory, and pathology assessment. We then excluded 1 participant due to age (> 19 years old), 3 due to insufficient sample quantity, and 1 due to inconclusive fibrosis biopsy score, for a final sample of 88 children with biopsy-proven NAFLD.

As a comparison group, we selected control participants from three completed or ongoing studies at Emory University and Children's Healthcare of Atlanta. This included (1) 16 healthy participants from an ongoing, cross-sectional, observational study

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of children being evaluated for suspected eosinophilic esophagitis (the EoE study); (2) 16 healthy participants from a previously completed, randomized, controlled fructose challenge trial (baseline samples only) (the Sweet Bev study)[21]; and (3) 34 healthy participants from an ongoing, cross-sectional, observational, data biorepository study of environmental exposures and childhood health (the PEACH study). All selected participants had a stored blood sample (at −80°C) available for analysis were 2-18 years old, had no chronic disease based on medical chart review, had alanine aminotransferase (ALT) ≤ 50 U/L for boys and ≤ 44 U/L for girls (two times the upper limit of cutoffs from the SAFETY study[22]), and, if the participant was obese, had recent clinical testing to rule out hepatic steatosis (≤ 5.5% based on magnetic resonance imaging [MRI]). One participant was excluded from the final analysis due to an insufficient sample, for a total of 65 healthy participants. Written informed consent was provided by all participants 18 years or older. For participants < 18 years, parents/guardians provided written informed consent, and participants provided verbal assent (if 6-10 years) or written assent (if 11-17 years). These protocols were approved by the institutional review boards at Emory University (Atlanta, GA) and Children's Healthcare of Atlanta (Atlanta, GA).

COVARIATE ASSESSMENT

Basic demographics, including age, sex, and race/ethnicity, and anthropometric measurements, including height in centimeters and weight in kilograms, were obtained at the time of liver biopsy and/or by medical chart review. Age and sex-adjusted body mass index (BMI) *z*-scores and percentiles were calculated using Centers for Disease Control and Prevention 2000 growth charts.[23] Weight category was derived from BMI percentiles, and participants were categorized with overweight if BMI > 85th and obesity if BMI > 95th percentile.

HISTOLOGICAL ASSESSMENT

Liver biopsies were clinically indicated and processed according to the local clinical routine, which included staining with hematoxylin and eosin and Masson's trichrome. For the purpose of this study, each section was independently and blindly reassessed by expert pathologists. Fibrosis was evaluated using the NASH Clinical Research Network (NASH CRN) staging system (0 = none; 1 = perisinusoidal or periportal; 1A = mild, zone 3, perisinusoidal; 1B = moderate, zone 3, perisinusoidal; 1C = portal/periportal; 2 = perisinusoidal and portal/periportal; 3 = bridging fibrosis; and 4 = cirrhosis),[24] and the Ishak staging system (0 = no fibrosis; 1 = fibrous expansion of some portal area; 2 = fibrous expansion of most portal areas; 3 = fibrous expansion of most portal areas with occasional bridging; 4 = fibrous expansion of most portal areas with marked bridging; 5 = marked bridging occasional nodules; and 6 = cirrhosis).[25] For the primary analysis, we used the Ishak staging system to dichotomize participants with NAFLD as with advanced fibrosis (Ishak score ≥ 3) or none/mild fibrosis (Ishak score ≤ 2) due to its seven-tired staging system, which provides a finer distinction of fibrosis changes and structural remodeling than other scoring systems, and allowed us to identify more participants with advanced fibrosis (n = 8). However, we also performed a sensitivity analysis to assess whether results were similar if we dichotomized participants with NAFLD based on NASH CRN fibrosis scores (advanced fibrosis = scores ≥ 3; and none/mild fibrosis = scores ≤ 2). Liver biopsies were also scored based on other histopathological features, including grade of steatosis (0 ≤ 5%; 1 = 5%-33%; 2 = 34%-66%; and 3 ≥ 66%), lobular inflammation (0 = no foci; 1 = two or fewer foci per ×200 field; 2 = two to four foci per ×200 field; and 3 = four or more foci per ×200 field), portal inflammation (0 = none; 1 = mild; and 2 = more than mild), and hepatocyte ballooning (0 = none; 1 = few; and 2 = many).

BIOMARKER QUANTIFICATION

Blood samples were collected before liver biopsy and stored at −80°C until use. Biochemical markers that were assessed were the liver enzymes ALT and aspartate aminotransferase (AST), which were measured by standard methods at the Children's Healthcare of Atlanta clinical laboratory. A neo-epitope marker specific for the assessment of the ADAMTS-2 (ADAM metallopeptidase with thrombospondin type 1 motif 2)–released N-terminal pro-peptide of type III collagen was assessed in serum samples by the use of PRO-C3, a competitive enzyme-linked immunosorbent assay developed by Nordic Bioscience (Herlev,

Denmark), as previously described.[26] CTX-I and N-MID, surrogate markers of bone turnover, associated with growth spurt in boys and girls,[27,28] were assessed in serum samples using the Roche Elecsys platform (Basel, Switzerland) at Nordic Bioscience.

STATISTICAL ANALYSIS

Descriptive statistics were performed to assess the characteristics of the sample overall and according to NAFLD group. Continuous variables were assessed for normality using histograms and the Shapiro-Wilk test, and nonparametric variables were reported as medians and interquartile ranges (IQRs). Otherwise, means and SDs were reported for continuous variables, and counts and frequencies were reported for categorical variables. Differences between NAFLD and control participants were assessed by t-tests for parametric variables and Mann-Whitney *U* test for nonparametric variables and chi-square test for categorical variables. The relationship between PRO-C3 levels in childhood and adolescence with participant characteristics and the bone biomarkers CTX-I and N-MID were examined with Spearman correlation for continuous variables and Kruskal-Wallis one-way analysis of variance for categorical variables. All analyses were performed in the full sample set and stratified by sex and by NAFLD status. Scatter plots were created to visualize trends in PRO-C3 according to these variables using the *ggplot2* package in R. Trend lines were created using linear regression (method = "lm") or nonparametric local regression (method = "loess"). To examine whether PRO-C3 levels differed in participants with advanced fibrosis, we constructed linear regression models with fibrosis stage (advanced fibrosis, none/mild fibrosis, or healthy participants) as the independent variable. Stepwise adjustment for covariates was performed as follows: Model 1 was adjusted for age, sex, race/ethnicity, and BMI *z*-score; model 2 was adjusted for model 1 covariates plus CTX-I; and model 3 was adjusted for model 1 covariates and N-MID. Due to skewed distributions, PRO-C3, CTX-I, and N-MID were all log-transformed before analyses. Results are therefore reported as the back-transformed least-squares means (LS-means) and 95% confidence intervals (CIs) for PRO-C3 in each fibrosis group. All statistical analyses were performed using SAS Statistical Software (version 9.4; SAS Institute Inc., Cary, NC), and all figures were created using R statistical software (version 3.4.4; R Foundation for Statistical Computing, Vienna, Austria[29]).

Results

SAMPLE CHARACTERISTICS

The characteristics of the sample overall and according to NAFLD group are summarized in Table 1. Participants with NAFLD, compared with healthy participants, were older (mean ± SD: 13.9 ± 2.9 vs. 12.0 ± 4.4 years, respectively), had a higher mean BMI *z*-score (3.1 ± 0.6 vs. 0.8 ± 1.1), and a higher percentage of males (72% vs. 39%) and Hispanic race/ethnicity (64% vs. 32%). ALT and AST were also higher in participants with NAFLD (median [IQR]: 90.5 [72.0, 129.2] vs. 18.0 [15.0, 22.0] for ALT and 44.5 [34, 80] vs. 18.5 [16.3, 23.8] for AST, respectively).

TRENDS IN PRO-C3 LEVELS IN CHILDHOOD AND ADOLESCENCE

Medians and IQRs for PRO-C3 according to categorical participant characteristics are given in Table 2. PRO-C3 levels were similar in participants with NAFLD (median [IQR]: 20.6 [15.8, 25.9] ng/mL) versus healthy participants (19.0 [13.8, 26.0] ng/mL), as well as by sex and race/ethnicity (Table 2). However, PRO-C3 was significantly lower in older adolescents ≥ 15 years old (median [IQR]: 16.4 [13.0, 21.2] ng/mL) versus children ≤ 10 years old (22.9 [18.1, 28.4] ng/mL) or 11-14 years old (22.4 [18.3, 31.2] ng/mL) ($P < 0.001$ for both), but did not differ when comparing children ≤ 10 years old versus 11-14 years old (Table 2). This pattern of findings is also visualized in Fig. 1, which shows that PRO-C3 was approximately stable among participants ≤ 14 years old, but declined among older adolescents (ages ≥ 15 years old). Among healthy participants only, PRO-C3 was also significantly lower in participants with overweight/obesity (16.3 [13.3, 20.3] ng/mL) versus normal weight (20.6 [16.3, 31.6] ng/mL) based on BMI percentiles (Table 2). To examine whether PRO-C3 levels were related to pubertal growth, we tested for correlations and constructed scatter plots to examine the relationships between PRO-C3 and the bone biomarkers CTX-I (bone resorption) and N-MID (bone formation) as continuous variables. This revealed positive correlations of PRO-C3 with CTX-I ($r = 0.64$;

TABLE 1. CHARACTERISTICS OF THE FULL SAMPLE AND STRATIFIED BY NAFLD GROUP

	Full Sample (n = 153)	Healthy (n = 65)	NAFLD (n = 88)	*P* Value*
Age (years), mean (SD)	13.1 (3.7)	12.0 (4.4)	13.9 (2.9)	0.012
Age category, n (%)				
≤10 years old	36 (23.5%)	20 (30.8%)	16 (18.2%)	0.056
11-14 years old	73 (47.7%)	24 (36.9%)	49 (55.7%)	
≥15 years old	44 (28.8%)	21 (32.3%)	23 (26.1%)	
Male sex, n (%)	88 (57.5%)	25 (38.5%)	63 (71.6%)	<0.001
Race/ethnicity, n (%)				
Non-Hispanic White	44 (28.8%)	21 (32.3%)	23 (26.1%)	<0.001
Non-Hispanic Black	28 (18.3%)	22 (33.8%)	6 (6.8%)	
Hispanic	77 (50.3%)	21 (32.3%)	56 (63.6%)	
Asian	4 (2.6%)	1 (1.5%)	3 (3.4%)	
BMI *z*-score, mean (SD)	2.1 (1.4)	0.8 (1.1)	3.1 (0.6)	<0.001
BMI category, n (%)				
Normal weight	36 (23.5%)	36 (55.4%)	0 (0.0%)	<0.001
Overweight	9 (5.9%)	8 (12.3%)	1 (1.1%)	
Obesity	108 (70.6%)	21 (32.3%)	87 (98.9%)	
CTX-I (ng/mL), median (IQR)	1.2 (0.9, 1.7)	1.3 (1.0, 1.7)	1.2 (0.9, 1.6)	0.492
N-MID (ng/mL), median (IQR)	56.2 (29.6, 80.7)	59.3 (31.1, 95.1)	55.5 (27.6, 75.0)	0.132
ALT (U/L), median (IQR)†	66.0 (19.5, 101.8)	18.0 (15.0, 22.0)	90.5 (72.0, 129.2)	<0.001
AST (U/L), median (IQR)†	33.0 (21.0, 53.2)	18.5 (16.2, 23.8)	44.5 (34.0, 80.0)	<0.001

**P* values calculated by Kruskal-Wallis t-test for continuous variables and chi-square test for categorical variables.
†Eleven control participants were missing values for ALT and AST.

P < 0.001) and N-MID (*r* = 0.62; *P* < 0.001) in the full sample, and when stratified by NAFLD group and/or sex (Fig. 2 and Supporting Table S1).

PRO-C3 LEVELS ACCORDING TO FIBROSIS STAGE AMONG CHILDREN WITH AND WITHOUT NAFLD

We next examined whether PRO-C3 levels differed in children with NAFLD and advanced fibrosis (Ishak score ≥ 3) compared to children with NAFLD and none/mild fibrosis (Ishak score < 3) and healthy children. The characteristics of the sample according to fibrosis status are given in Table 3. Compared to participants with none/mild fibrosis, those with advanced fibrosis had a higher percentage of boys (75% vs. 71.2%) and Hispanic race/ethnicity (87.5% vs. 61.2%), and were younger (mean [SD]: 11.4 ± 2.2 years vs. 14.2 ± 2.9 years). ALT was also higher in advanced fibrosis (median [IQR]: 188 [93.2, 316]) versus none/mild fibrosis (89.5 [68, 122.2]) (Table 3). In unadjusted analyses, PRO-C3 levels differed significantly by fibrosis group (*P* = 0.007 based on Kruskal-Wallis tests) (Fig. 3). Specifically, PRO-C3 was highest in advanced fibrosis (median [IQR]: 29.8 ng/mL [26.9, 35.0]) compared with none/mild fibrosis (19.3 ng/mL [15.6, 24.7]; *P* = 0.001) and healthy participants (19.0 ng/mL [13.8, 26.0]' *P* = 0.001) in pairwise comparisons using Dunn's test. In multivariable-adjusted linear regression models, these differences were attenuated, but remained significant after adjusting for age, sex, and BMI *z*-score in model 1. When we added CTX-I (in model 2) or N-MID (in model 3) as covariates in linear regression models, differences in PRO-C3 were only significant for comparisons of participants with NAFLD and advanced fibrosis versus healthy controls, but were attenuated for comparisons of participants with NAFLD and advanced versus NAFLD and none/mild fibrosis (Table 4).

PRO-C3 LEVELS ACCORDING TO OTHER LIVER FIBROSIS SCORES OR OTHER HISTOPATHOLOGICAL VARIABLES

We conducted a sensitivity analysis to examine differences in PRO-C3 if we instead used the

TABLE 2. ESTIMATES FOR PRO-C3 (NG/ML) ACCORDING TO PARTICIPANT CHARACTERISTICS FOR THE FULL SAMPLE AND STRATIFIED BY NAFLD GROUP

	Full Sample (n = 153)		Healthy (n = 65)		NAFLD (n = 88)		Boys (n = 88)		Girls (n = 65)	
	Median (IQR)	*P**	Median (IQR)	*P**	Median (IQR)	*P**	Median (IQR)	*P**	Median (IQR)	*P**
Overall	19.6 (15.0, 25.9)	—	19.0 (13.8, 26.0)	—	20.6 (15.8, 25.9)	—	20.6 (16.6, 26.8)	—	18.3 (13.7, 25.9)	—
Age category										
≤ 10 years old†	22.9 (18.1, 28.4)	**<0.001**	21.4 (18.0, 27.5)	**0.001**	25.9 (20.9, 28.4)	**0.001**	21.4 (18.4, 28.4)	**0.038**	24.6 (18.7, 27.8)	**<0.001**
11-14 years old†	22.4 (18.3, 31.2)	**<0.001**	20.3 (16.3, 33.4)	**0.002**	22.7 (19.6, 28.2)	**0.004**	22.6 (20.0, 30.6)	**0.001**	19.4 (15.4, 30.1)	**0.017**
≥ 15 years old	16.4 (13.0, 21.2)	ref	13.6 (12.2, 18.8)	ref	18.1 (14.6, 22.8)	ref	18.2 (14.7, 23.2)	ref	13.8 (12.7, 18.5)	ref
Race/ethnicity‡										
Non-Hispanic White	19.6 (14, 23.4)	ref	17.6 (15.0, 22.1)	ref	21.0 (15.6, 24.8)	ref	19.9 (16.5, 22.2)	ref	17.5 (12.7, 25.4)	ref
Non-Hispanic Black	18.8 (14.8, 32.4)	0.20	28.0 (15.4, 36.0)	0.11	17.5 (15.4, 18.1)	0.22	19.6 (17.7, 32.0)	0.25	18.4 (12.9, 35.5)	0.51
Hispanic	19.7 (15.4, 25.0)	0.45	16.3 (13.0, 20.3)	0.43	22.5 (16.0, 28.2)	0.30	21.9 (16.5, 27.7)	0.29	18.4 (13.3, 24.1)	0.95
BMI category§										
Normal weight	20.6 [16.3, 31.6]	ref	20.6 [16.3, 31.6]	ref	—	—	20.5 (17.8, 31.6)	ref	21.1 (14.6, 31.3)	ref
Overweight/obesity	19.3 [14.8, 25.0]	0.25	16.3 [13.3, 20.3]	**0.048**	—	—	20.6 (16.3, 25.6)	0.50	18.1 (13.7, 24.1)	0.30

**P* values were estimated from Dunn's test comparing PRO-C3 for each category to a reference category (indicated by "ref"). Bold values indicate $P < 0.05$.
†There were no significant differences in PRO-C3 for participants with NAFLD versus healthy controls ($P = 0.40$) and for boys versus girls ($P = 0.10$). There were no significant differences in PRO-C3 for ≤ 10 years old versus 11-14 years old for the full sample or any subgroup.
‡Due to small sample size (n = 4), Asian race/ethnicity was excluded from pairwise comparisons.
§All participants with NAFLD were overweight or obese; therefore, no pairwise comparisons were performed.

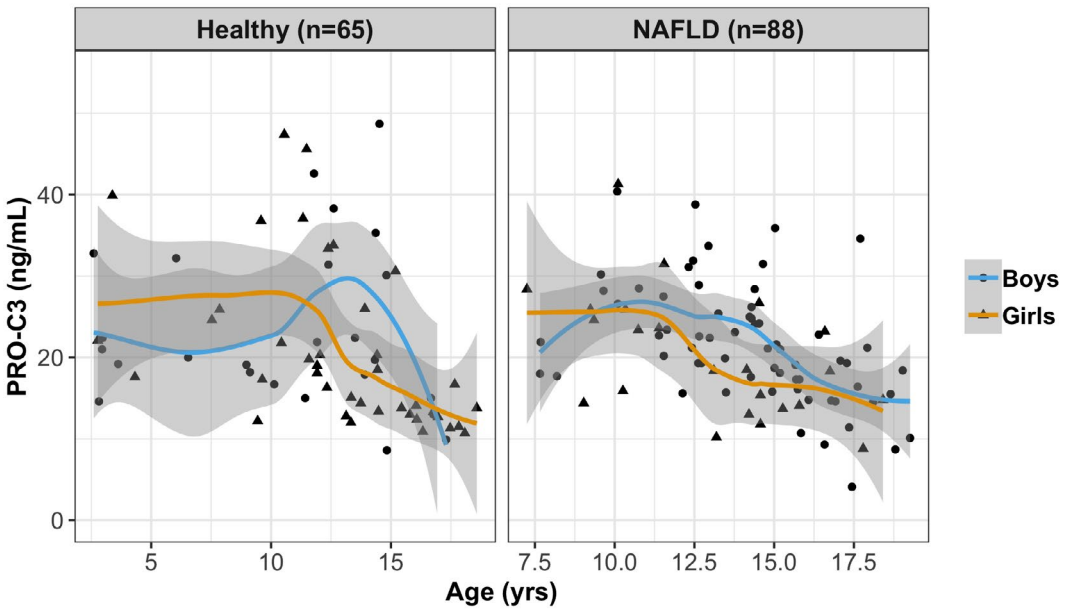

FIG. 1. Trends in PRO-C3 levels according to participant age among participants with NAFLD (n = 87) and healthy participants (n = 65). Within each plot, trends are stratified by sex, as indicated by circles (boys) and triangles (girls) and blue (boys) versus yellow (girls) trend lines. All trend lines were estimated by locally weighted smoothing regression (method = "loess") in *ggplot2*. One participant with an outlier value for PRO-C3 (61.6 ng/mL) was excluded from the plot.

NASH-CRN fibrosis scores to dichotomize participants (advanced fibrosis = scores ≥ 3; none/mild fibrosis = scores ≤ 2). Most participants were dichotomized into the same category as with Ishak scores, except for 2 participants who were no longer categorized as having advanced fibrosis based on NASH-CRN scores. As shown in Supporting Fig. S1, PRO-C3 still differed significantly by fibrosis group in unadjusted analyses (P = 0.043 based on Kruskal-Wallis tests), such that PRO-C3 was highest in advanced fibrosis (median [IQR]: 28.3 [24.4, 32.4] ng/mL) compared with none/mild fibrosis (19.5 [15.6, 24.9] ng/mL; P = 0.018) and healthy participants (19.0 [13.8, 26.0] ng/mL; P = 0.012). Results were also similar in stepwise linear regression models when using NASH-CRN scores compared with the Ishak scores, except differences between participants with advanced fibrosis versus none/mild fibrosis were slightly attenuated (Supporting Table S2).

In an exploratory analysis, we also examined whether PRO-C3 varied according to other histology scores assessed by liver biopsy. As shown in Fig. 4, PRO-C3 did not differ according to grade of steatosis (P = 0.90), lobular inflammation (P = 0.86), portal inflammation (P = 0.20), or hepatocyte ballooning (P = 0.64) based on Kruskal-Wallis tests. Medians and IQRs for PRO-C3 according to each histological variable are given in Supporting Table S3.

Discussion

In this study, we measured PRO-C3, the N-terminal propeptide of type III procollagen that detects formation of type III collagen, in children with and without biopsy-proven NAFLD and across a range of ages and race/ethnicities. Our main finding was that PRO-C3 levels in childhood are strongly associated with age, whereby levels were highest in participants ages ≤ 10 years old and 11-14 years old, and then declined among older adolescents 15 years or older (approximately aligning with the end of the pubertal growth spurt). Consistent with a link between PRO-C3 levels and pubertal growth, we found that PRO-C3 was strongly and positive correlated both with CTX-I and N-MID, which are markers of bone remodeling that likely reflect tissue turnover during growth, so-called modeling. As a potential biomarker of advanced fibrosis in pediatric NAFLD, PRO-C3 was considerably higher in children with NAFLD and

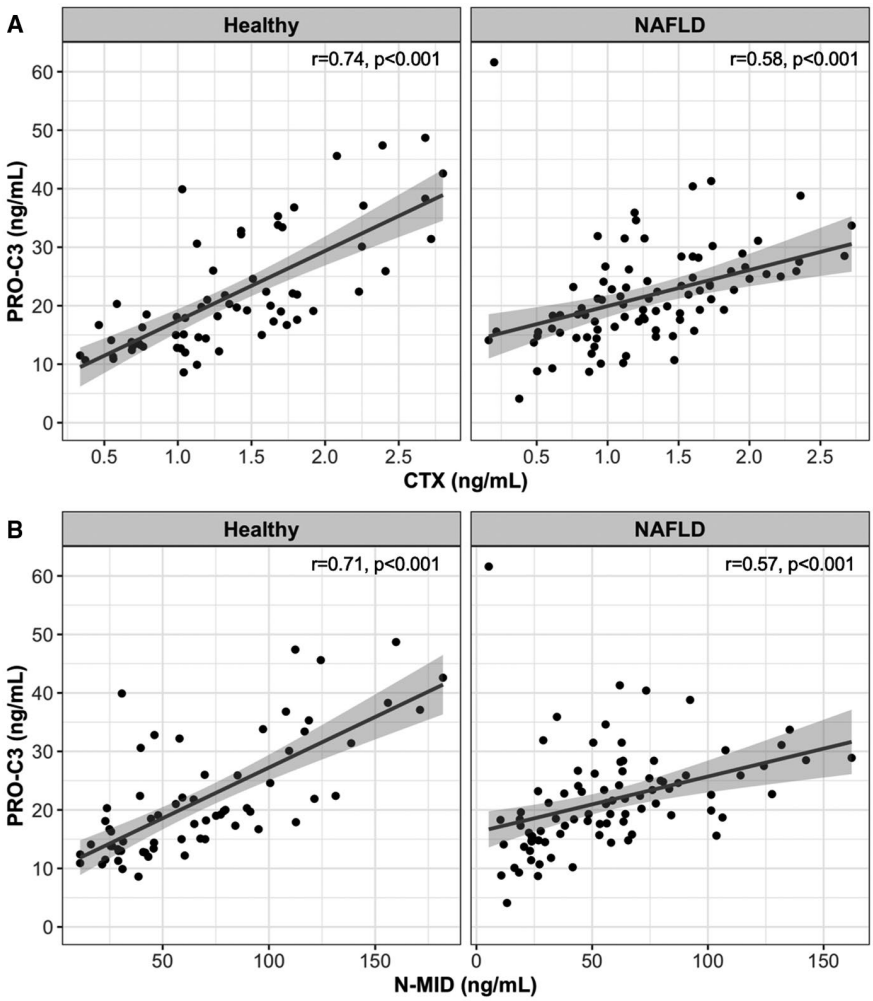

FIG. 2. Scatter plots showing correlations between PRO-C3 and the bone biomarkers CTX-I (A) and N-MID (B) among participants with NAFLD (n = 88) and healthy participants (n = 65). Correlation coefficients and *P* values were calculated by Spearman correlation. All trend lines were estimated by linear regression (method = "lm") in *ggplot2*.

advanced fibrosis (based on Ishak scores) compared to those with NAFLD and none/mild fibrosis or healthy children; however, these differences were weakened after adjusting for these bone biomarkers. This suggests that, although in adults PRO-C3 is considered to be a marker of active fibrogenesis,[10,11,12,14] growth spurts confound the interpretation of PRO-3 levels in children. Therefore, PRO-C3 may have limited utility as a diagnostic tool for advanced fibrosis in pediatric NAFLD until later adolescence. Because all of the participants with NAFLD and advanced fibrosis were under 15 years old in the present study, we could not test this hypothesis using a stratified analysis; therefore, future studies with larger sample sizes and a wider age range are needed to compare the utility of PRO-C3 as a biomarker of advanced fibrosis across different pubertal stages.

Remodeling of the ECM is a critical biological process throughout life for maintaining healthy tissue, but can become disturbed in certain disease states, including fibrosis; thus, ECM biomarkers have gained in interest as potential biomarkers of fibrogenesis in adults.[11] However, increased tissue turnover skewed toward tissue build-up also occurs during periods of growth and hormonal changes, which represents a critical confounder in the relationship between ECM-related biomarkers and fibrosis in certain subgroups, especially youth. Studies both in mice[30] and in humans[31] have shown that biomarkers of type III procollagen vary with age, such that levels are

TABLE 3. CHARACTERISTICS OF THE SAMPLE ACCORDING TO FIBROSIS STAGE BASED ON ISHAK SCORING

	Advanced Fibrosis* (n = 8)	None/Mild Fibrosis* (n = 80)	Healthy (n = 65)	$P^{\dagger}$
Age (years), mean (SD)	11.4 (2.2)	14.2 (2.9)	12.0 (4.4)	0.002
Male sex, n (%)	6 (75.0%)	57 (71.2%)	25 (38.5%)	<0.001
BMI *z*-score, mean (SD)	2.9 (0.5)	3.1 (0.6)	0.8 (1.1)	<0.001
Race/ethnicity, n (%)				
White	1 (12.5%)	22 (27.5%)	21 (32.3%)	<0.001
Black	0 (0.0%)	6 (7.5%)	22 (33.8%)	
Hispanic	7 (87.5%)	49 (61.2%)	21 (32.3%)	
Asian	0 (0.0%)	3 (3.8%)	1 (1.5%)	
BMI category, n (%)				
Normal weight	0 (0.0%)	0 (0.0%)	36 (55.4%)	<0.001
Overweight	0 (0.0%)	1 (1.2%)	8 (12.3%)	
Obesity	8 (100.0%)	79 (98.8%)	21 (32.3%)	
PRO-C3 (ng/mL), median (IQR)	29.8 (26.9, 35.0)	19.3 (15.6, 24.7)	19.0 (13.8, 26.0)	0.007
CTX-I (ng/mL), median (IQR)	1.7 (1.5, 2.1)	1.2 (0.9, 1.6)	1.3 (1.0, 1.7)	0.019
N-MID (ng/mL), median (IQR)	73.7 (62.1, 102.2)	52.1 (26.8, 71.8)	59.3 (31.1, 95.1)	0.019
ALT (U/L), median (IQR)[‡]	188.0 (93.2, 316.5)	89.5 (68.0, 122.2)	18.0 (15.0, 22.0)	<0.001
AST (U/L), median (IQR)[‡]	80.0 (53.2, 141.0)	43.0 (33.5, 69.2)	18.5 (16.2, 23.8)	<0.001

*Categorized based on Ishak scoring. Advanced fibrosis was defined by Ishak score ≥ 3.
†*P* values calculated by Kruskal-Wallis t-test for continuous variables and chi-square test for categorical variables.
‡Eleven control participants were missing ALT and AST values.

constant during early childhood, with a peak during puberty, and then gradually fall into late adolescence/adulthood. In addition, Kehlet et al. described changes in collagen turnover biomarkers in 617 healthy adults, and PRO-C3 levels increased among post-menopausal women (> 60 years old) compared with men,[32] which suggests a potential impact of hormonal status on ECM remodeling; however, it should be noted that this study did not adjust for potential confounding variables, such as weight changes. In the present study, we confirmed several of these findings in a diverse sample of children with and without NAFLD, and expanded on this research by showing a strong relationship between trends in PRO-C3 and levels of bone resorption biomarkers, CTX-I and N-MID.

Regarding the utility of PRO-C3 as a biomarker for advanced fibrosis in pediatric NAFLD, we did observe higher PRO-C3 in participants with advanced fibrosis in analyses adjusting for age, sex, and BMI *z*-score, which aligns with studies in adults showing that PRO-C3 is an independent predictor of fibrosis stage in patients with NAFLD,[10] as well as other liver diseases including hepatitis B and C.[33,34] In fact, the median values that we observed for PRO-C3 in children with NAFLD and advanced fibrosis (29.8 ng/mL) and none/mild fibrosis (19.3 ng/mL) were similar to values reported in adults with NAFLD (31.7 and 17.9 ng/mL, respectively[10]). We cannot comment on longitudinal changes in PRO-C3 in this cohort, but findings from adult studies suggest that PRO-C3 has promise as a biomarker both for identifying patients with fibrotic liver disease at baseline, who may benefit the most from antifibrotic therapy, as well as for measuring improvements in liver fibrosis in response to treatment.[15,35] The utility of PRO-C3 as a response biomarker in pediatric NAFLD will need to be evaluated in future studies.

However, it is critical to note that these differences in PRO-C3 between participants with advanced versus none/mild fibrosis were considerably weakened when adjusting for either of the bone remodeling biomarkers CTX-I and N-MID. Therefore, caution should be exercised in using PRO-C3 as a fibrosis biomarker in youth with NAFLD before the pubertal growth spurt is complete. Relative to the literature, studies testing other noninvasive fibrosis prediction scores in pediatric NAFLD have also been largely unsuccessful,[8,36] and this may relate to confounding factors that are specific to childhood, such as the substantial growth that occurs, and differences in the

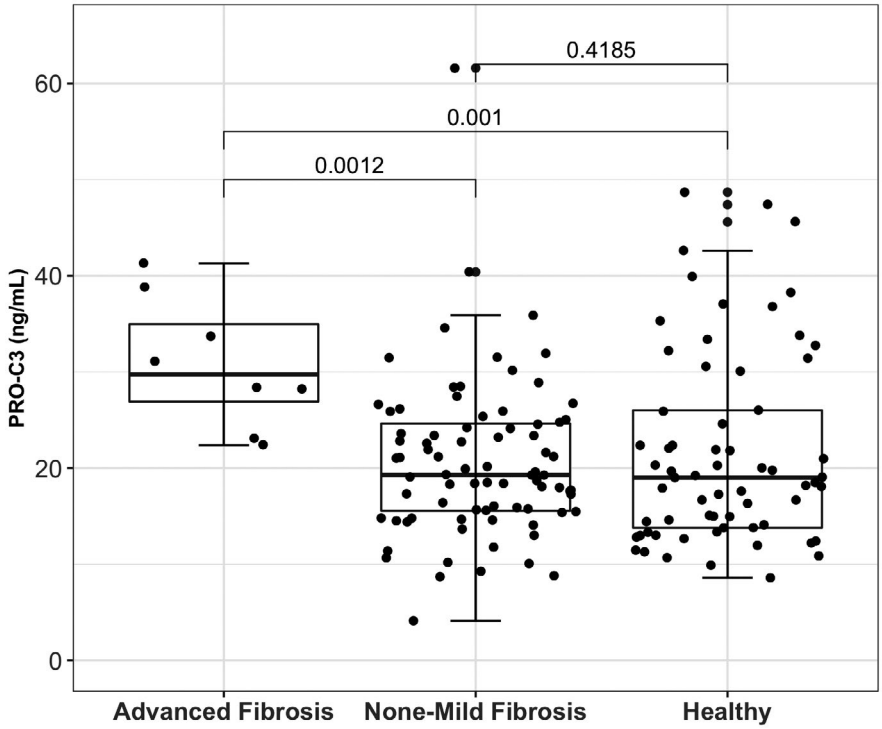

FIG. 3. PRO-C3 levels according to fibrosis stage based on Ishak scores in children and adolescents with NAFLD and healthy controls. Participants were categorized with advanced fibrosis based on fibrosis scores ≥ 3 (n = 8) or none/mild fibrosis based on scores ≤ 2 (n = 80). *P* values were calculated for pairwise comparisons using the nonparametric Dunn's test.

TABLE 4. ESTIMATES FOR PRO-C3 ACCORDING TO FIBROSIS STAGE BASED ON ISHAK SCORES IN STEPWISE LINEAR REGRESSION MODELS

	Advanced Fibrosis* (n = 8)	None/Mild Fibrosis* (n = 80)		Healthy (n = 65)	
Model	Mean (95% CI)†	Mean (95% CI)†	*P*‡	Mean (95% CI)†	*P*‡
1: Adjusted for age, sex, BMI *z*-score	28.5 (21.6, 37.6)	20.3 (18.2, 22.8)	**0.020**	18.4 (16.1, 21.0)	**0.010**
2: Model 1 + CTX-I	26.4 (20.5, 34.0)	21.1 (19.1, 23.5)	0.09	18.1 (16.0, 20.5)	**0.015**
3: Model 1 + N-MID	26.4 (20.9, 33.9)	21.0 (19.0, 23.3)	0.08	18.2 (16.1, 20.5)	**0.014**

*Categorized based on Ishak scoring. Advanced fibrosis was defined by Ishak score ≥ 3.
†LS-means and 95% CIs estimated from multivariable-adjusted linear regression after back-transformation.
‡*P* values estimated from pairwise comparisons of LS-means and 95% CIs for log-transformed PRO-C3 by fibrosis group, compared with advanced fibrosis as the reference category. Bold values indicate $P < 0.05$.

pathophysiology of NAFLD in children compared with adults. In particular, studies have shed light on the potential for more rapid progression of liver damage in children with NAFLD[3,4,37] and have described an alternate histological pattern of NASH (type 2 or pediatric-type NASH), which is common in younger children and characterized by moderate-high steatosis with portal inflammation and fibrosis, but little or no ballooning.[38,39] The exact mechanism to explain these differences in children versus adults remain unclear, but it may not be a coincidence that participants with advanced fibrosis in our pediatric NAFLD cohort tended to be younger than those with none/mild fibrosis, and may therefore reflect a unique population of children who develop progressive NAFLD at a young age, which will require different biomarkers than adults due to the simultaneous onset of the pubertal growth spurt.

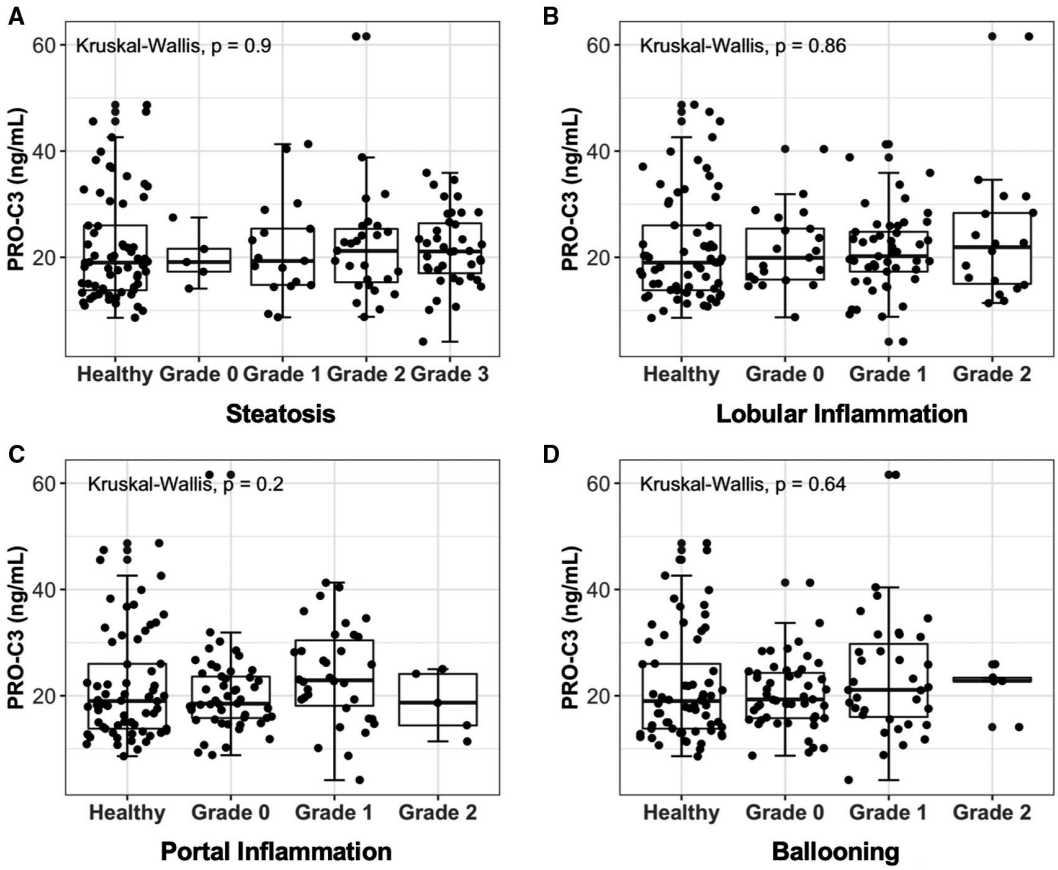

FIG. 4. PRO-C3 levels in children and adolescents with NAFLD and healthy controls according to other histological variables: steatosis (A), lobular inflammation (B), portal inflammation (C), and ballooning (D). *P* values were calculated by Kruskal-Wallis one-way analysis of covariance tests. Medians and IQRs for PRO-C3 according to grade of each histological variable are provided in Supporting Table S3.

As a proof-of-concept study, there are some limitations that should be noted. First, we are not able to state any causal relationship between PRO-C3 and other outcomes due to the cross-sectional design, and longitudinal, interventional studies will be needed to assess this. Second, our small sample size, and especially the limited number of children with advanced fibrosis in NAFLD, may affect our statistical power in detecting smaller effect sizes. Although we attempted to mitigate this by pooling several cohorts, the data from these cohorts were collected and stored over different time periods, which may have introduced additional variability in our estimates and CIs. We also could not definitively confirm that *all* healthy participants were without steatosis or fibrosis, because some were selected from previously completed studies that did not include MRI or liver biopsy assessments; however, we are sufficiently confident that the inclusion criteria for healthy participants limited this possibility. Furthermore, all participants with NAFLD had assessments of steatosis and fibrosis by liver biopsy; thus, this limitation did not affect comparisons within the NAFLD group. Finally, we did not have consistent assessments of pubertal stage on all participants, and therefore could not assess this variable as a covariate with PRO-C3, which would be needed to confirm potential confounding by the pubertal growth spurt. We also did not have assessments of other potential confounding variables, such as diabetes/prediabetes status, which may be relevant in the understanding of fibrogenesis in pediatric NAFLD. In addition, there were considerable differences in terms of demographics and other traits, such as adiposity measured by BMI *z*-score, between the participants with NAFLD and healthy participants. Although we attempted to control for this in analyses, it is possible that residual confounding was an issue.

In conclusion, we showed that, while PRO-C3 levels were considerably higher in children with NAFLD and advanced fibrosis, this relationship may be subject to the strong confounding effects of age and the pubertal growth spurt. Further validations studies in other pediatric cohorts with NAFLD, ideally with larger sample sizes and/or with longitudinal, repeated measurements of PRO-C3, will be needed in order to elucidate whether PRO-C3 may be a predictor of future fibrosis progression and/or of treatment response.

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